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Quantitative determination in urine of hippuric acid and *m*- or *p*-methylhippuric acid, metabolites of toluene and *m*- or *p*-xylene

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Ogata, M., Tomokuni, K., and Takatsuka, Y. (1969). *Brit. J. industr. Med.*, **26**, 330-334. **Quantitative determination in urine of hippuric acid and *m*- or *p*-methylhippuric acid, metabolites of toluene and *m*- or *p*-xylene.** Improved and more specific methods for the quantitative determination of hippuric and *m*- and *p*-methylhippuric acids in urine are described. The acids were extracted from urine with ether/ethanol, which was dried with silica gel, or with ethyl acetate. After removing the solvent by evaporation coloured azlactones were formed by reaction with *p*-dimethylaminobenzaldehyde in acetic anhydride (DAB, only usable after ether/ethanol extraction) or benzenesulphonyl chloride in pyridine, and the absorbances were measured. The sensitivities were about 4 μg./ml. of urine using DAB, and 20 μg./ml. of urine using benzenesulphonyl chloride reagent. Separation of hippuric and methylhippuric acids was achieved by paper and thin-layer chromatography before estimation. A spot test is also described.

Toluene and xylene are widely used, both together and separately, as organic industrial solvents. It is quite difficult to estimate the average concentrations of toluene or xylene in the air in places where they are used as these concentrations vary considerably within a working day. It is more practicable, therefore, to calculate the average concentrations inhaled by workers from the metabolites excreted in urine.

Toluene administered to animals is first oxidized to benzoic acid, which conjugates with glycine to form hippuric acid. According to El Masry, Smith, and Williams (1956), about 75% of the toluene administered to rabbits is excreted as hippuric acid. Thus it appears likely that most of the toluene inhaled by workers will also be excreted in the urine as hippuric acid, the quantitative analysis of which should give an estimate of the quantity of toluene inhaled by workers. The only previous check on this suggestion was carried out by von Oettingen, Neal, and Donahue (1942), who determined urinary hippuric acid from volunteers exposed to toluene in concentrations over 100 p.p.m. in their laboratory. However, they used a crystallization-titration method which is not sensitive enough for the quantitative analysis of urinary hippuric acid from workers exposed to small amounts of toluene.

There are three different isomers of xylene, *o*-, *m*-, and *p*-, and a mixture commonly called 'xylol' of which 75 to 85% is *m*-xylene. *In vivo* these isomers are oxidized to *o*-, *m*-, and *p*-toluic acids, of which the *m*- and *p*-isomers conjugate with glycine and are excreted in the urine as the corresponding toluylglycines, *i.e.*, as *m*- and *p*-methylhippuric acids. *o*-Toluic acid, however, is thought to be excreted as the ether glucuronide (Williams, 1959).

In order to determine urinary hippuric acid we

tried to use our modification (Ogata, Sugiyama, and Moriyasu, 1962) of Gaffney's (Gaffney, Schreier, Di Ferranti, and Altman, 1954) paper chromatographic method and Pagnotto and Lieberman's (1967) ultra-violet absorption method. However, we found that in Gaffney's method the coloration differed with the kind of filter paper used, and a standard solution had to be applied to each paper. Pagnotto and Lieberman's method is not specific to hippuric acid, because there is light absorption from uric acid and other substances. To overcome these difficulties, we first extracted the hippuric acid from urine with ether/ethanol or ethyl acetate, dried the extract, and converted the hippuric acid to its coloured azlactone with *p*-dimethylaminobenzaldehyde or benzenesulphonyl chloride. The resulting method proved to be specific and reproducible. It could also be used to measure *m*- and *p*-methylhippuric acids, the metabolites of *m*- and *p*-xylene. Methylhippuric acids could be separated from hippuric acid by paper and thin-layer chromatography.

Materials and methods

Urine specimens

Specimens were obtained from healthy men not exposed to toluene or xylene and from volunteers exposed for 3 hours to various concentrations (100 to 200 p.p.m.) of toluene or to a mixture of toluene (67 p.p.m.) and xylene (83 p.p.m.).

Analytical procedures

Silica gel method Urine (1 ml.) was placed in a stoppered tube, its pH was adjusted to 2·0 with HCl, and it was nearly saturated with NaCl (0·3 g.). Hippuric acid was extracted twice with 2 ml. of ethyl ether/ethanol (9:1 by vol.). The ether extract was diluted 10 times with ethyl ether/ethanol (9:1). The extract gave a negative test for urea with *p*-dimethylaminobenzaldehyde. One millilitre of the diluted extract was poured onto 0·5 g. silica gel in a test tube, and the solvent was removed by evaporation under reduced pressure. The silica gel containing the hippuric acid was heated in an oil bath at 135°C. for 3 minutes with 4% *p*-dimethylaminobenzaldehyde solution in acetic anhydride containing a few crystals of sodium acetate. The azlactone formed was extracted twice successively with 4 ml. and 3 ml. of ethanol, and the optical density was read at 460 nm. Standard curves were obtained by using aqueous solutions of hippuric acid and *m*- or *p*-methylhippuric acid solutions (0·2 to 1·5 mg./ml.) instead of urine in the above method.

Benzenesulphonyl chloride method The extraction of hippuric acid from urine was carried out with ethyl acetate, but ether/ethanol may be used; 0·1 or 0·2 ml. of extract was put into a test tube and dried under reduced pressure. The specimen was taken up in 0·5 ml. of pyridine solution, 0·2 ml. of benzenesulphonyl chloride was added, and the mixture was allowed to stand for 30 minutes at room temperature. It was then diluted with 4·3 ml. chloroform and the absorbance was read at 380 nm. against a pyridine-benzenesulphonyl chloride blank.

The spot method Hippuric acid solutions (0·25, 0·5, 1·0, 1·5, 2·0, 2·5, and 3·0 mg./ml. in water) were prepared. Extracts as from the silica gel method were obtained, and 0·2 ml. of each extract was placed in the dimple of a porcelain tile. After drying, 0·1 ml. pyridine and 0·04 ml. benzenesulphonyl chloride were added. By adding 0·1 ml. of chloroform the colour was stabilized for 12 to 13 minutes against only 7 minutes in its absence. The azlactone colour from urine extracts was compared with that from the standard solutions.

Separation of hippuric acid and methylhippuric acids by chromatography

Paper chromatography The ethyl acetate extract of urine or standard (0·2 ml.) was developed on filter paper (40 × 40 cm. Toyo Filter Paper Company, No. 51) with toluene-acetic acid-water (100:50:2·3) for 6 hours at 20°C.

The paper was dried, and the spots were developed by spraying with *p*-dimethylaminobenzaldehyde reagent and by heating at 135°C. for 1 minute. The R_f values for hippuric acid, *m*-methylhippuric acid, and *p*-methylhippuric acid were 0·38, 0·45, and 0·43 respectively. An example using urine containing hippuric acid and methylhippuric acid obtained from a volunteer exposed to a mixture of toluene and xylene is shown in Figure 1A. To estimate the hippuric acids the spots were cut out, cut into small pieces, and extracted with ethanol (Ogata *et al.*, 1962), and the absorbance was determined as above.

Alternatively, the positions of the spots were found by illuminating the filter paper with ultra-violet light of maximum intensity at 253·6 nm. The spots were marked, cut out, and extracted twice with 3 ml. of ethanol, and the colour with *p*-dimethylaminobenzaldehyde reagent on silica gel was developed as before.

A colour may alternatively be developed by reaction with benzenesulphonyl chloride as described above.

Thin-layer chromatography The plates were prepared using activated silica gel G as absorbent. Ethyl acetate extract, 0·12 ml., was spotted on each thin-layer plate, 3 cm. from the lower margin and 3 cm. in width, and the chromatogram was developed with toluene-acetic acid-water (100:50:2·3 by vol.) for one hour at 20°C. The solvent was allowed to evaporate, and the plates were sprayed with *p*-dimethylaminobenzaldehyde reagent, and heated at 135°C. for 3 minutes to develop the colour. The azlactones were extracted with 4 ml. and 3 ml. of ethanol successively, and the absorbance was measured at 460 nm. The appearance of the plates before extraction is shown in Figure 1B.

The paper and thin-layer chromatograms may also be developed using *n*-butanol-acetic acid-water as described in our previous report (Ogata *et al.*, 1962).

Absorption spectrum The absorption spectrum of the azlactone was measured with a Beckman DK-2A spectrophotometer.

Results

Absorption spectra and absorbances of azlactones

The absorption maxima of the azlactones from

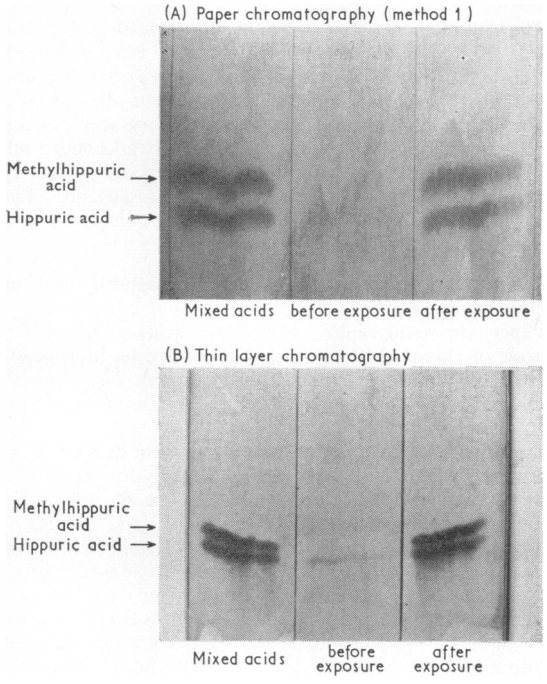

FIG. 1. (A) Paper and (B) thin-layer chromatograms of hippuric acid and *m*-methyl-hippuric acid in the urine of volunteers exposed to mixtures of toluene and *m*-xylene.

hippuric acid and *m*- and *p*-methylhippuric acids were all at 460 to 470 nm. The molar extinctions were $1{\cdot}5 \times 10^4$ for hippuric acid azlactone and $1{\cdot}4 \times 10^4$ for *m*- and *p*-methylhippuric acids azlactone at 460 nm. The absorption spectra of the colours given by the benzenesulphonyl chloride method showed two main absorption peaks at 425 nm and 445 nm with every acid. On standing, however, the maximum at 425 nm gradually decreased and shifted to 380 nm with a change in colour from red to orange. Since no further change in absorbance occurred, 380 nm was the wavelength selected for the quantitative measurement of absorbance, as described by Umberger and Fiorese (1963). Again hippuric acid gave slightly higher absorbances than the methylhippuric acids. The *p*-dimethylaminobenzaldehyde method was more sensitive than the benzenesulphonyl chloride one.

Recovery tests

Recoveries of hippuric acid added to urine ranged from 94 to 100% (Table 1). The variations probably resulted from differences in the efficiencies of extraction, and not from incomplete colour formation. As shown in Fig. 2, the benzenesulphonyl chloride and silica gel methods gave very similar results.

TABLE 1

RECOVERY OF ADDED HIPPURIC ACID OR *m*-METHYLHIPPURIC ACID IN NORMAL HUMAN URINE USING *p*-DIMETHYLAMINOBENZALDEHYDE IN SILICA GEL OR BENZENESULPHONYL CHLORIDE AS REAGENT

Reagent	*Hippuric acid added (μg.)*	*Recovery (%)*
DAB in silica gel ..	20	95
	50	96
	100	94
	150	90
Average	—	94
BSC	20	105
	50	96
	100	96
	150	97
Average	—	99
Reagent	*m-Methylhippuric acid added (μg.)*	*Recovery (%)*
DAB in silica gel ..	20	95
	50	96
	100	92
	150	92
Average	—	94
BSC	20	105
	50	96
	100	100
	150	96
Average	—	100

DAB = *p*-dimethylaminobenzaldehyde.
BSC = benzenesulphonyl chloride.

The spot test was used on 40 specimens of urine, and the results were compared with those obtained by the benzenesulphonyl chloride method. There were no serious discrepancies (Table 2).

Simultaneous determination of hippuric and methylhippuric acids

When the colours were developed on paper the absorbances after extraction were only about one third of those found in the methods not involving chromatography, and varied from paper to paper. When the spots were located by ultra-violet light and the colours were developed after elution, the absorbances were nearly as high as in the methods not involving chromatography. When, however, the colours were developed on the thin-layer chromatograms before elution, the absorbances were as high as when the silica gel method was used on eluates

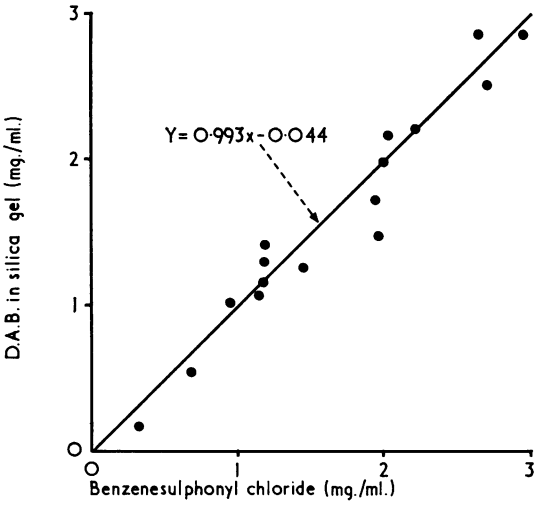

FIG. 2. Relationship between concentration of hippuric acid measured by the benzenesulphonyl chloride method and by the *p*-dimethylaminobenzaldehyde (DAB) in silica gel method.

from paper. Most of the hippuric acid in the spot was converted to azlactone; and thin-layer chromatography proved to be rapid and highly reproducible.

The results of recovery tests are shown in Table 3. The recoveries were uniformly high. It is obvious from the results that more accurate assays can be obtained by comparing results with those obtained using standard solutions which are treated exactly like the urine specimens. It was shown that the various methods gave very similar results on the urines of volunteers exposed to toluene and xylene (Figs. 3 and 4).

TABLE 3

RECOVERY OF ADDED HIPPURIC ACID AND *m*-METHYLHIPPURIC ACID FROM NORMAL HUMAN URINE AFTER CHROMATOGRAPHY

Reagent	*Hippuric acid added (μg.)*	*Recovery (%)*
DAB in silica gel ..	20	95
	50	104
	100	103
	150	102
Average	—	101
BSC	20	105
	50	96
	100	93
	150	100
Average	—	99
Reagent	*m-Methylhippuric acid added (μg.)*	*Recovery (%)*
DAB in silica gel ..	20	95
	50	86
	100	97
	150	98
Average	—	94
BSC	20	105
	50	98
	100	96
	150	91
Average	—	98

DAB = *p*-dimethylaminobenzaldehyde.
BSC = benzenesulphonyl chloride.

TABLE 2

SPOT TEST FOR HIPPURIC ACID COMPARED WITH THE BENZENESULPHONYL CHLORIDE METHOD

Urine, hippuric acid (mg./ml.) by BSC method	*Screening (spot) test reactions, urine hippuric acid (mg./ml.)*						
	0·2-0·4	*0·5-0·9*	*1·0-1·4*	*1·5-1·9*	*2·0-2·9*	*≥ 3·0*	*Total*
0·2-0·4	5	—	—	—	—	—	5
0·5-0·9	—	6	3	—	—	—	9
1·0-1·4	—	—	5	—	—	—	5
1·5-1·9	—	—	—	5	—	—	5
2·0-2·9	—	—	—	3	5	—	8
≥ 3·0	—	—	—	—	2	6	8
Total	5	6	8	8	7	6	40

BSC = benzenesulphonyl chloride.
Perfect agreement would be shown if all the numbers except totals appeared on the diagonal.

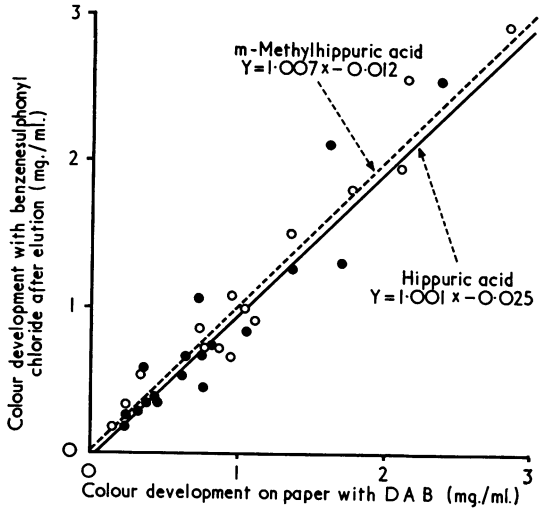

FIG. 3. Relationship between the concentrations of hippuric acid (●) and *m*-methylhippuric acid (○) found by spraying the papers with *p*-dimethylaminobenzaldehyde and those found by the benzenesulphonyl chloride method after chromatography.

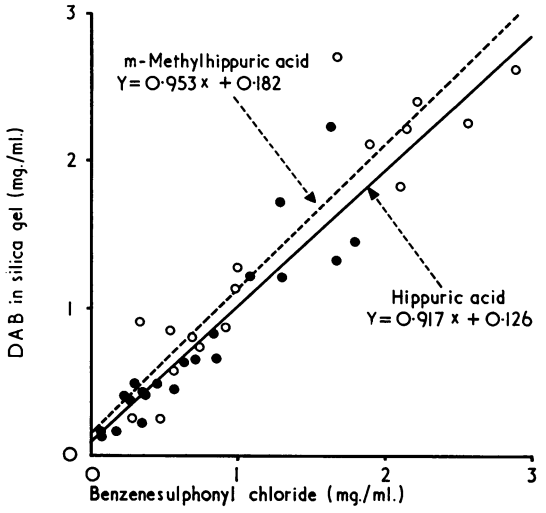

FIG. 4. Relationship between the concentrations of hippuric acid (●) and *m*-methylhippuric acid (○) measured by two methods after elution.

Discussion

The methods for hippuric and methylhippuric acids given here all involve an extraction stage. Ether/ethanol could always be used as the extracting solvent, as it does not extract urea. Ethyl acetate was a more convenient solvent but does extract some urea. It could, therefore, not be used with *p*-dimethylaminobenzaldehyde, which reacts with urea, except after chromatography, which separates the hippuric acid from urea. It could, however, be used with benzenesulphonyl chloride as this did not give a colour with urea.

The determination of hippuric and methylhippuric acids by coloured azlactone formation after extraction from urine with ether/ethanol or ethyl acetate is a specific method, and therefore gives lower concentrations in the urine of non-exposed persons (0·3 mg./ml., Ogata *et al.*, 1962) than the ultra-violet spectrophotometric method of Pagnotto and Lieberman (1967) which gives 0·8 mg./ml. as it is not specific for glycine conjugates. Uric acid in particular also absorbs ultra-violet light. When the urine contains both hippuric acid and methylhippuric acid these are given as the sum of both, because the absorbances are similar, but the acids can be isolated by paper or thin-layer chromatography and determined separately.

The analytical sensitivity of the crystallization-titration method used by von Oettingen *et al.* (1942) is only about 1 mg. Taking the least detectable amounts by our methods as those giving absorbances of 0·05, the *p*-dimethylaminobenzaldehyde method was sensitive to 4 μg. and the benzenesulphonyl chloride method to 20 μg. The less sensitive method, that using benzenesulphonyl chloride, is the simpler, as there is no stage involving silica gel or heating. It is still adequate to detect exposure to low concentrations of toluene and xylene.

All the methods were highly reproducible except the one in which the spots were developed on the filter paper (Gaffney *et al.*, 1954). The absorbance then varied with each filter paper, due to differences in spraying or heating, and a standard solution containing 50 μg. each of hippuric acid and methylhippuric acid had to be applied to each filter paper for comparison.

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